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CHARACTERIZATION AND PROBIOTIC POTENTIALS OF LACTIC ACID BACTERIA ISOLATED FROM INGESTA OF SELECTED RUMINANTS

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Studies were carried out to isolate and screen lactic acid bacteria with probiotic potentials and amylolytic activities from the ruminal ingesta of cow and goat. Ninety isolates obtained from the three abattoirs were divided into the following species: *Lactobacillus plantarum*, *L. fermentum*, *L. pentosus*, *L. brevis*, *L. buchneri*, *L. collinoides*, and *Leuconostoc mesenteroides*. The isolates were screened for probiotic potentials and amylolytic activities. Four isolates possessed probiotic potentials: *L. plantarum* CA3b, *L. fermentum* GA2d, *L. plantarum* GA1d, and *L. plantarum* GA3e. *Lactobacillus plantarum* CA3b, *L. fermentum* GA2d, and *L. plantarum* GA3e survived pH 2.5 and 3.5 after 180 min. *Lactobacillus plantarum* GA1d had the highest tolerance of 82.44% and 71.03% to 0.3% and 1% bile salts concentrations, respectively. *Lactobacillus plantarum* CA3b hydrolysed 3% starch with an average zone of clearance of 10.0 mm, while *L. plantarum* GA1d hydrolysed 2% with a zone of 9.5 mm. These two strains also possessed bacteriocin activities against *E. coli*, and were characterised based on their reactions to pH, temperature, and enzymes treatments. Ruminal ingesta of cow and goat harbour lactic acid bacteria possessing amylolytic and probiotic potentials, which can be exploited in the fermentation of feedstock, conferring health benefits and improved performance to these ruminants.

Keywords: ruminal ingesta, lactic acid bacteria, probiotics, amylolytic activity

Ruminants are mammals of the order Artiodactyla (DAVIS & MILNER, 2009). They have four chambered stomach made up of the reticululum, rumen, omasum, and abomasum (DEHORITY & TIRABASSO, 2000; DAVIS & MILNER, 2009). Ruminant animals such as cow, sheep, goat, and deer, however, do not synthesize fibre digesting enzymes, but they have formed a symbiotic relationship with ruminal microorganisms that do (DEHORITY & TIRABASSO, 2000).

The microbial quality of the ingesta (ingested food) depends to a large extent on the quality and composition of feed stuff (BELANCHE et al., 2012; UYENO et al., 2015). The microbial flora of the ingesta is a function of the nature, quality, and composition of the animals' feed, which is dominated by complex polysaccharide and starch degrading microbiota. Lactic acid bacteria (LAB) among other bacteria and fungi found in the ingesta of ruminants have been isolated and documented (BELANCHE et al., 2012; UYENO et al., 2015). These are added as bacteriocin producing cultures or by the addition of pure or semi-pure bacteriocin preparations. Several LAB have been isolated from intestinal tracts of ruminants. These include species of the genera *Lactococcus*, *Leuconostoc*, *Streptococcus*, *Pediococcus*, *Ruminococcus*, and *Lactobacillus* (FRAGA et al., 2013; UYENO et al., 2015).

Lactic acid bacteria constitute an integral part of the healthy gastrointestinal (GI) microecology and are involved in the host metabolism. A number of mechanisms work to prevent harmful bacteria from growing and attaching to the intestinal epithelium, examples include

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production and secretion of antimicrobial agents such as bacteriocins and organic acids, and adherence via competition for the binding sites interfering with pathogens (REID et al., 2001; WANG et al., 2010).

The aim of this study was to screen various isolates of LAB from ruminal ingesta of cow and goat for the possible exploitation as potential probiotics and starter culture(s) for feedstock of ruminant diet.

1. Materials and methods

1.1. Collection of samples

Samples were obtained from healthy cattle (cow and goat) from three selected abattoirs; the University of Ibadan Teaching and Research farm and Bodija and Moniya cattle markets in Ibadan, South West Nigeria. Three cows and three goats were sampled from each cattle farm at each sampling time. The sampling was carried out at five different times from each farm. Samples were collected immediately after evisceration from the reticulo-rumen with the assistance of a qualified veterinarian assigned to each abattoir.

1.1.1. Isolation of LAB. The samples obtained from the cows and goats were homogenized separately. A total of 10 g per 100 ml (w/v) of the homogenized sample were stored in normal saline for 5 h. A 10-fold serial dilution was made using 10 ml stock sample into 90 ml distilled water. These were cultured for 48 h in de Man Rogosa Sharpe agar (MRS) using pour plate method under anaerobic conditions. They were further sub-cultured in MRS agar under anaerobic conditions until pure isolates were obtained. Pure cultures were maintained as stocks in MRS broth at -4°C , with 15% glycerol.

1.2. Biochemical characterization of the LAB isolates

1.2.1. Carbohydrates fermentation. Biochemical characterization was carried out on the isolates by assimilation of carbohydrates using API 50CH strips and API 50CHL medium (Biomérieux, Marcy Etoile, France) according to the instructions of the manufacturer. The results from the assimilation profiles were identified using the Apiweb™ software version 5.1.

1.3. Probiotic potentials of the selected LAB isolates

1.3.1. Resistance to low pH. The ability of the isolates to tolerate low pH was determined according to the method of VINDEROLA and REINHEIMER (2003). The mixture was enumerated for the presence of viable cells after 1, 2, and 3 h by measuring the optical density (OD) at 560 nm wavelength using a spectrophotometer (Jenway UV-Vis 5210, Essex, UK), and the results were compared with a control.

1.3.2. Bile tolerance and hydrophobicity tests. Bile salt tolerance and hydrophobicity were assayed by the method of VINDEROLA and REINHEIMER (2003). Hydrophobicity was calculated as the percentage decrease in the $(\text{OD}_{600\text{nm}})$ of the initial aqueous bacterial suspension due to cells partitioning into a hydrocarbon layer. The percentage of cell surface hydrophobicity (%H) of the strain adhering to xylene was calculated using the equation: $\text{H}\% = [(\text{A}_0 - \text{A}) / \text{A}_0 \times 100]$.

1.4. Starch hydrolysis

Surface dried plates of starch based MRS agar [1, 2, and 3% starch (w/v)] (SANNI et al., 2002) were streaked with 18 h old culture of LAB and incubated at 30 °C for 3 days. The plates were flooded with Gram's iodine solution for 15 to 30 min and examined for clear zones around the growth for assessment of amylolytic activity.

1.5. Genotypic characterization of the selected amylolytic LAB isolates

The genomic DNA of the selected amylolytic LAB strains were extracted using the modified lysozyme-heat lysis method. The DNA quantity and purity were determined using a spectrophotometer at absorbance reading of 260 nm (Nanodrop, Thermo Scientific, USA). The partial 16S rRNA nucleotide sequence was amplified by PCR using universal primers designed for lactic acid bacteria, forward was (5' – GAG TTT GAT CCT GGC TCA G – 3'), while the reverse was (5' – AGA AAG GAG GTG ATC CAG CC – 3') (BANWO et al., 2012). The PCR products were cleaned using spin columns and subsequently sequenced in a commercial laboratory (Beijing, China).

1.6. Antimicrobial activities of selected LAB isolates

The antibacterial activity of the selected isolates against indicator pathogens *Listeria monocytogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* were determined. The isolates were grown in MRS broth (pH 6.5) inoculated with 1% of an overnight culture and incubated at 37 °C for 18–24 h. After incubation, cells were removed from the growth medium by centrifugation (6000×g for 20 min, 4 °C). The antimicrobial spectrum of the inhibitory substances from LAB was determined using the agar well diffusion method (AWD) and observed for zones of inhibition (SCHILLINGER & LÜCKE, 1989).

1.6.1. Screening for bacteriocin producing isolates. The cell-free supernatants of LAB were adjusted to pH 6.0–6.5 using 5 N NaOH to exclude the antimicrobial effect of organic acids. Inhibitory activity of hydrogen peroxide was eliminated by the addition of catalase at a final concentration of 1.0 mg ml⁻¹. The catalase-treated samples were incubated for 2 h at 37 °C. After incubation, the treated and neutralized cell-free supernatants were then tested for antagonistic activity against indicator bacteria by the AWD (SCHILLINGER & LÜCKE, 1989).

1.6.2. Characterization of bacteriocin. The bacteriocins were subjected to treatment at different temperatures, pH, and enzymes. The enzymes were added to 1 ml of neutralized cell free culture supernatant to a final concentration of 2.0 mg ml⁻¹ in all cases. The tubes were incubated at 37 °C for 1 h and the residual bacteriocin activities were determined by measuring the zones of inhibition on agar plates (BANWO et al., 2013). Untreated cell free supernatants were used as control.

1.7. Statistical analyses

Each experiment described in this research study was done in replicates and data obtained from the study were analysed using descriptive statistics and analysis of variance. The differences among means were separated using Duncan's multiple range test. Significant differences were accepted at P>0.05. The statistical software used was SPSS 16.5 for Windows 7.

2. Results and discussion

2.1. Characterization of LAB isolates using API 50CHL Kit

Ninety LAB isolates were biochemically characterized into the following species of *Lactobacillus plantarum* (32), *L. fermentum* (25), *L. pentosus* (15), *L. brevis* (4), *L. buchneri* (4), *L. collinoides* (3), and *Leuconostoc mesenteroides* (7) (data not shown). The assessment of biochemical and physiological properties of lactic acid bacteria maybe a good method for phenotypic characterization, but it is not adequate (MOHAMMED et al., 2011; BANWO et al., 2012). There were differences in the species of LAB isolates from each ruminant in a particular sampling site, which agreed with the report of BELANCHE and co-workers (2012), that the different species isolated from the rumen (reticulo-rumen) is largely dependent on nutrition and health status of the ruminant. Selection of the isolates was based on the desirable properties of probiotic potentials and amyolytic activities.

2.2. Resistance to low pH

The results of colony forming units (CFU ml⁻¹) are shown in Table 1. In this study, it was observed that some strains tolerated pH 2.5 and 3.5 for three hours, which is in agreement with previous reports of probiotic LAB (BANWO et al., 2013). Some authors proposed that strains intended for probiotic purposes should be screened according to their tolerance to pH 2.5 in an HCl-acidified culture medium for 3 hours (WANG et al., 2010; MOHAMMED et al., 2011).

Table 1. Survival count of selected LAB strains on exposure to pH 3.5 and 2.5 (log₁₀ CFU ml⁻¹)

Isolate	pH			
	2.5 (0 min)	2.5 (after 180 min)	3.5 (0 min)	3.5 (after 180 min)
<i>L. plantarum</i> CA3b	4.76±0.03 ^a	2.29±0.34 ^b	5.17±0.01 ^c	3.11±0.21 ^b
<i>L. plantarum</i> GA1d	4.76±0.13 ^a	–	4.22±0.43 ^d	–
<i>L. fermentum</i> GA2d	4.65±0.03 ^b	2.74±0.15 ^a	5.97±0.31 ^a	4.11±0.19 ^a
<i>L. plantarum</i> GA3e	4.08±0.03 ^d	2.17±0.14 ^c	5.11±0.01 ^c	3.11±0.12 ^b

Values are means of three independent experiments (mean ± SD)

–: No visible growth/loss of viability; C: cow; G: goat; A: animal; 1: University of Ibadan Teaching and Research farm; 2: Bodija cattle farm; 3: Moniya cattle farm; b;d:e: sampling periods

Values with the same letters in superscript along a column are not significantly different (P>0.05).

2.3. Bile salt tolerance

The isolates from this study grew at 0.3% bile salt concentration, however, tolerance varied at higher concentrations of 0.5% and 1.0% (Fig. 1). Though the bile concentrations in the gastrointestinal tract vary, the mean concentration is 0.3% w/v (VINDEROLA & REINHEIMER 2003). Interestingly, the strains stable at low pH were also bile salt tolerant. This is similar to the result obtained by WANG and co-workers (2010), who reported that the strains of *L. buchneri*, *L. brevis*, and *L. plantarum* were resistant to 0.3% bile salt at pH 4.0.

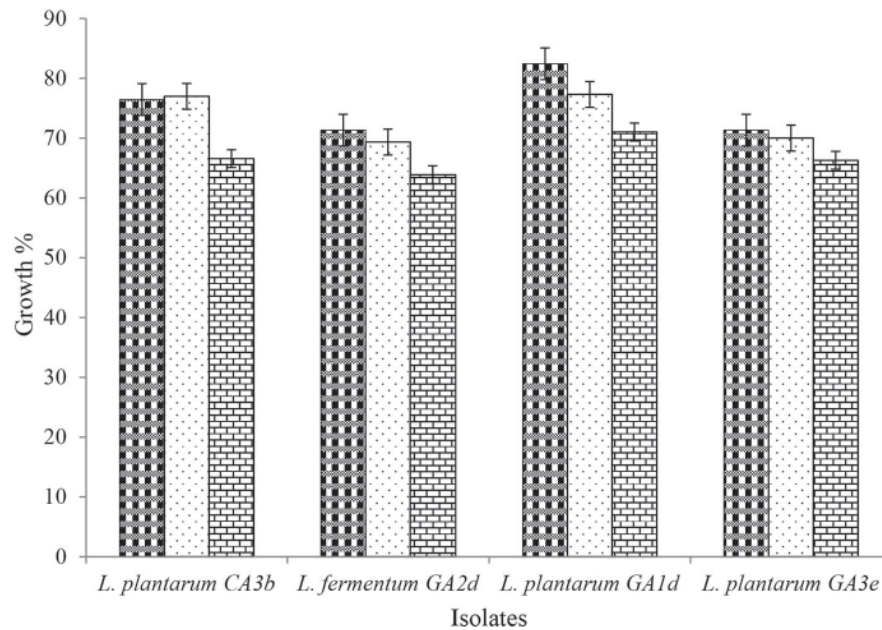


Fig. 1. Percentage bile salt tolerance of selected LAB strains to 0.3%, 0.5%, and 1.0% bile salt concentrations. C: cow; G: goat; A: animal; 1: University of Ibadan Teaching and Research farm; 2: Bodija cattle farm; 3: Moniya cattle farm; a,b,c,d,e: sampling periods.

Bars represent the mean and standard deviations of three independent determinations

☒: 0.30%; ☒: 0.50%; ☒: 1.00%

2.4. Bacterial adhesion to hydrocarbon assay (BATH) and starch hydrolysis activities of the strains

The selected LAB isolates were screened for their cell surface hydrophobicity as shown in Table 2. The gastrointestinal tract, especially the small intestine, is a dynamic environment, and the flow of digesta washes out any bacterium unable to counter the flow either by rapidly multiplying or by attaching itself to intestinal surfaces (MOHAMMED et al., 2011; GITIKA & TIWARI, 2013; UYENO et al., 2015). Amylase is an enzyme, which breaks down starch into simple sugars. Consequently, only few lactic acid bacteria possess the ability to hydrolyse starch to produce amylase. The selected isolates demonstrated amylase activity by showing clear zones of hydrolysis on starch based MRS agar plates. Only one isolate, *L. plantarum* CA3b displayed the ability to hydrolyse 3% starch concentration (Table 2). Very few amyolytic lactic acid bacteria (ALAB) are reported from traditional fermentation of food and feedstock (SANNI et al., 2002; REDDY et al., 2008). Potential probiotic LAB able to hydrolyse starch will be an added advantage in the fermentation of feedstock with added health benefit. The two isolates that possessed amyolytic potentials were identified using 16S rRNA gene sequencing as *L. plantarum* CA3b and *L. plantarum* GA1d with accession numbers MF099794 and MF099795, respectively.

Table 2. Cell surface hydrophobicity and starch hydrolysis assay of the selected LAB strains

Isolates	Hydrophobicity (%)			Starch concentration (w/v)		
	Initial value	Final value	Adherence	1	2	3
<i>L. plantarum</i> CA3b	1.04±0.40	0.31±0.03	70.19±0.07 ^c	+*	+*	+
<i>L. plantarum</i> GA1d	1.06±0.40	0.30±0.02	71.70±0.06 ^c	+*	+	-
<i>L. fermentum</i> GA2d	1.31±0.02	0.21±0.03	83.97±0.08 ^d	+	-	-
<i>L. plantarum</i> GA3e	1.06±0.04	0.33±0.01	68.87±0.07 ^b	+	-	-

Values are expressed as the mean ± standard deviation of three independent determinations

Values with the same letters in superscript along a column are not significantly different (P>0.05). C: cow; G: goat; A: animal; 1: University of Ibadan Teaching and Research farm; 2: Bodija cattle farm; 3: Moniya cattle farm; b;d;e: sampling periods

-: No visible amyolytic activity (no visible zone of clearance); +: Amyolytic activity; +*: Strong amyolytic activity. Experiments were carried out at 37 °C over 5 days

2.5. Antimicrobial activities, production and characterization of crude bacteriocin

Crude cell free culture supernatants of strains were tested against common pathogens as indicator microorganisms (Table 3). After neutralization of the cell free culture supernatants, four strains demonstrated antagonistic activities against *E. coli*. As previously reported, the antagonistic activities of LAB towards pathogens can be attributed to the production of bactericidal substances like bacteriocins, organic acids, and hydrogen peroxide (TALPUR et al., 2012; FRAGA et al., 2013).

Table 3. Zones of inhibition (mm) of antimicrobial action of cell free culture supernatant obtained from the LAB strains against indicator microorganisms before and after neutralization

Isolates	ZI before neutralization Indicator microorganisms				ZI after neutralization
	<i>Escherichia coli</i>	<i>Listeria monocytogenes</i>	<i>Klebsiella pneumoniae</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>
<i>L. plantarum</i> CA3b	25.0±0.08 ^a	24.4±0.09 ^b	22.4±0.11 ^a	19.0±0.12 ^a	18.0±0.08 ^b
<i>L. plantarum</i> GA1d	24.0±0.09 ^b	21.3±0.11 ^c	16.3±0.20 ^d	17.0±0.21 ^c	18.3±0.08 ^b
<i>L. fermentum</i> GA2d	21.1±0.10 ^c	16.4±0.21 ^d	18.0±0.12 ^b	18.0±0.20 ^b	19.7±0.06 ^a
<i>L. plantarum</i> GA3e	23.1±0.10 ^c	22.2±0.11 ^c	17.1±0.15 ^c	18.0±0.20 ^b	-

Activity expressed as average zone of inhibition (mm). (P>0.05), C: cow; G: goat; A: animal; 1: University of Ibadan Teaching and Research farm; 2: Bodija cattle farm; 3: Moniya cattle farm; b;d;e: sampling periods. Each test was carried out at 30 °C for 18–24 hours. Values with the same letters in superscript along a column are not significantly different (P>0.05).

Tables 4 show the effect of pH, temperature, and enzyme treatments on bacteriocins produced by the amyolytic strains *L. plantarum* CA3b and *L. plantarum* GA1d against *E. coli*. The activity of the bacteriocins produced was not significantly affected by pH 4.0 to 8.0, but activity was highest at pH 6.0, while there was no activity at pH 9.0. Maximum activity was observed at pH 6.0, suggesting that compounds other than acids inhibited growth (BANWO et al., 2013).

Table 4. Effect of pH, temperature, and enzyme treatments on bacteriocins produced by *L. plantarum* CA3b and *L. plantarum* GA1d against *E. coli*

<i>L. plantarum</i> CA3b										<i>L. plantarum</i> GA1d				
pH	ZI (mm)	Temperature/Time	ZI (mm)	Enzyme	ZI (mm)	pH	ZI (mm)	Temperature/Time	ZI (mm)	Enzyme	ZI (mm)			
4	14.1	15 °C / 3 days	14.5	Proteinase K	-	4	12.2	15 °C / 3 days	15.5	Proteinase K	-			
6	17.3	15 °C / 5 days	13.9	Pepsin	-	6	15.4	15 °C / 5 days	14.6	Pepsin	-			
8	10.5	100 °C / 30 min	13.2	Amylase	16.2	8	12.5	100 °C / 30 min	13.4	Amylase	15.2			
9	-	100 °C / 60 min	11.0			9	-	100 °C / 60 min	12.5					

-: No activity; C: cow; G: goat; A: animal; 1: University of Ibadan Teaching and Research farm; 2: Bodija cattle farm; 3: Momiya cattle farm; b;d: sampling periods. Values are expressed as average zone of inhibition. Each test was carried out at 37 °C for 18–24 h

The bacteriocins were stable at 15 °C and 100 °C temperature for 30 and 60 min, but activity was lost at 121 °C for 15 min. This supports the findings of TODOROV and co-workers (2011), they observed that bacteriocin GP1 produced by *Lb. rhamnosus* had a remarkable stability over heat treatment even at the autoclaving temperature for 20 min. Heat stability of bacteriocins produced by lactic acid bacteria at 100 °C is important as a food biopreservative. Complete inactivation was observed after treatment of the cell-free supernatant with pepsin and proteinase K, which confirmed the proteinaceous nature of the active agent. Amylase treatment did not result in inactivation. This implies that carbohydrate moiety if existing in the culture supernatant is not significantly responsible for the inhibitory activity. Similar results were recorded by TODOROV and co-workers (2011) for bacteriocins produced by *L. plantarum* ST13BR. They observed that only proteolytic enzymes neutralized the activities of the bacteriocins.

3. Conclusions

The strains identified as *Lactobacillus plantarum* CA3b and *Lactobacillus plantarum* GA1d in this study produced bacteriocins and possessed amylolytic activities together, which is a very rare quality in the literature of lactic acid bacteria. Nature and composition of feedstock and other ecological factors could be associated with this factor. The lactic acid bacteria isolated possessed the generally regarded as safe status, which enable them to be employed as potential starter cultures for the fermentation of feedstock for enhanced performance.

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